Volume 269, number 1, 89–92 FEBS 08733 August 1990

Inhibition of oxygen toxicity by targeting superoxide dismutase to endothelial cell surface

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Received 8 Juni 1990

Since enzymes that degrade reactive oxygens, such as superoxide dismutase (SOD), are significantly lower in plasma than in intracellular compartments, cell surface membranes should be protected against hazardous oxygens particularly when animals are challenged with oxidative stress. To minimize oxygen toxicity on endothelial cell surface, a fusion gene consisting of cDNA coding human Cu^{2+}/Zn^{2+}-SOD and heparin-binding peptide was constructed and expressed in yeast. The resulting enzyme (HB-SOD) bound to a heparin-Sepharose column and cultured endothelial cells; binding was inhibited either by high NaCl concentrations or heparin. When injected intravenously, HB-SOD predominantly bound to vascular endothelial cell surface. Carrageenin-induced paw edema and cold-induced brain edema of the rat were markedly inhibited by a single dose of HB-SOD. These results suggest that superoxide radical and/or its metabolite(s) occurring at or near the outer surface of vascular endothelial cells might play a critical role in the pathogenesis of vasogenic edema.

Superoxide dismutase; Gene engineering; Oxygen toxicity; Heparin; Endothelial cell; Ischemic heart disease

1. INTRODUCTION

Oxygen stress is one of the major impacts on aerobic life. Since most cells are highly enriched with antioxidants and enzymes that degrade reactive oxygens [1,2], oxidative stress occurring inside cells can efficiently be minimized. Although similar enzymes are also present in plasma, such as extracellular SOD (EC-SOD) [3], their activities are significantly low. Thus, when tissues were challenged with high concentrations of extracellular reactive oxygens, membrane perturbation leading to cell injury might be triggered. Intravenously injected SOD decreases oxidative stress only briefly predominantly because of its rapid excretion in urine. EC-SOD has a specific binding site (consisting of 26 amino acids) for heparin-like proteoglycans [4] and seems to be a potent inhibitor of oxygen toxicity. However, large scale expression of recombinant EC-SOD sufficient for medical use has not been achieved yet presumably because of its glycoprotein nature [5].

To overcome such frustrating situations, a fusion gene encoding human Cu^{2+}/Zn^{2+}-SOD having a C-terminal heparin-binding domain (HB-SOD) was constructed and expressed in yeast. The present work demonstrates that HB-SOD specifically binds to heparin-like proteoglycans on endothelial cell surface and inhibites cold-induced brain edema and carrageenin-induced paw edema of the rat.

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2. MATERIALS AND METHODS

2.1. *Materials*

Conventional enzymes were obtained from Toyobo Co. Ltd (Osaka) and Takara Shuzo Co. Ltd (Kyoto). Evan's blue, xanthine, xanthine oxidase, bovine SOD were purchased from Sigma Co. Ltd (Sr. Louis). Carrageenin was from Zushi Chemical Co. Ltd (Tokyo). ^{125}I-Labeled Bolton-Hunter reagent (2.2 Ci/mol) was from New England Nuclear Co. Ltd. (Boston).

2.2. *Construction of a fusion gene encoding HB-SOD*

A fusion gene encoding HB-SOD consisting of human Cu^{2+}/Zn^{2+}-SOD and a C-terminal heparin-binding peptide with 26 amino acids similar to the heparin-binding domain of EC-SOD was constructed and expressed in yeast essentially as described previously [6]. Briefly, a full length of human SOD cDNA was obtained from a human placenta cDNA library [7]. A restriction site of *Eco*RI was made just before the initiation codon by site-directed mutagenesis [8]. About 450 bp *Eco*RI-*Sau*3AI fragment of SOD gene was subcloned into *Eco*RI-*Bam*HI sites of pBR322 and the constructed plasmid was denoted as pBRSOD. A nucleotide fragment, which encoded heparin binding peptide of EC-SOD with a stop codon and restriction sites of *Sau*3AI and *Sal*I, was constructed by annealing four synthetic nucleotides, 5'GATCTGCGGGCCCGGGATCTGGGAGCGCCAGGCGCGGGAGCACT3' (Fr. A), 5'TCTTGCGCTCTGAGTGCTCCCGCGCCTGGCGCTCCCAGATCCCGGGCCCGCA3' (Fr. B), 5'CAGAGCGCAAGAAGCGGCGGCGCGAGAGCGAGTGCAAGGCCGCCTGAG3' (Fr. C), and 5'TCGACTCAGGCGGCCTTGCACTCGCTCTCGCGCCGCCGCT3' (Fr. D) as described previously [9]. The constructed fragments were ligated with about 4.5 kb *Bam*HI-*Sal*I fragments of pBRSOD and the constructed plasmid was

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00145793/90/$3.50

denoted as pBRHBSOD. pBRHBSOD contained the HB-SOD gene encoding SOD having the heparin binding peptide with 26 amino acids on its C-terminal portion. About 0.5 kb *Eco*RI-*Sal*I fragment of pBRHBSOD was ligated with 8.0 kb *Eco*RI-*Sal*I fragment of a yeast expression vector pYHCC101 [10] and the constructed plasmid was denoted as pYHBS1. pYHBS1 contained *TRP1* as a selectable marker for yeast and HB-SOD gene in the plasmid was controlled by glyceraldehyde-3-phosphate dehydrogenase promotor in yeast. A yeast strain EH13-15 (*Mat α, trp1*) [11] was transformed by pYHBS1 as described [12]. Fig. 1 shows the diagram for the construction of HB-SOD gene and the structure of HB-SOD.

2.3. *Purification of HB-SOD*

The transformants were cultivated in Burkholder medium [13] and the expression of HB-SOD was confirmed immunologically using anti-SOD antibody; about 5% of cellular protein was found to be HB-SOD. Following heat treatment and chromatography on SP-Sephadex A-50, heparin-Sepharose and Sephadex G-75 columns, about 300 mg of HB-SOD was purified from 300 g of cells. Purification of HB-SOD by affinity chromatography on heparin-Sepharose was carried out essentially as described previously [14]; under physiological salt concentrations, HB-SOD, but not SOD, bound to the heparin-Sepharose column and was eluted by 1 M NaCl. Apparent molecular masses of 21 000 and 40 000 were obtained for HB-SOD by SDS-polyacrylamide gel electrophoresis and Sephadex G-75 column chromatography under nondenaturing conditions, respectively; specific activity of HB-SOD was 1500 unit/mg protein as determined by cytochrome *c* method [15]. Analysis by gas-phase sequencer suggested that the N-terminal amino acid of HB-SOD was also acetylated as was native SOD [16]. SOD and HB-SOD revealed similar stability to heat treatment; more than 90% of enzyme activity remained after treating with 70°C for 10 min. HB-SOD samples thus obtained were dissolved in phosphate buffered saline (pH 7.4) and were stored at −80°C until use; under such conditions, the enzyme can be stored at least for 6 months without decreasing its catalytic activity.

2.4. *Binding to endothelial cell surface*

Endothelial cells were isolated from freshly obtained bovine aorta by trypsin digestion, cultured in Eagle's minimum essential medium (MEM) containing 5% fetal calf serum, and were used for experiments 2 days after confluent as described previously [17]. Binding of ^{125}I-labeled HB-SOD and SOD to endothelial cell surface was determined at 4°C in Eagle's MEM as described previously [18].

2.4. *In vivo experiments*

Male Sprague-Dawley rats, 200–210 g, were used for experiments after fasting for 15 h. Under light ether anesthesia, animals were subcutaneously injected with 0.1 ml of carrageenin dissolved in saline solution (10 mg/kg). The volume of the paw was measured by using a Plethysmometer (Unicom Model TK-101).

Under pentobarbital anesthesia (50 mg/kg), cold-induced brain edema was induced essentially as described previously [19].

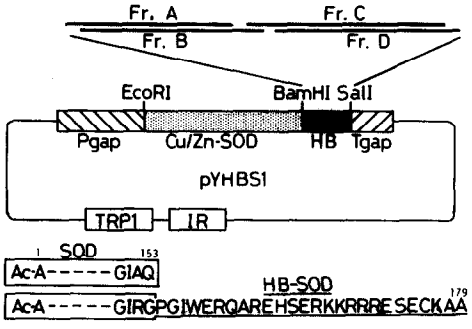

Fig. 1. Construction of plasmid pYHBS1 and structure of HB-SOD. Spotted area represents human Cu^{2+}/Zn^{2+}-SOD cDNA and solid area (HB) represents cDNA for the heparin-binding peptide. Open area represents the *TRP1* and *IR* genes of plasmid pYHBS1. Thin line represents plasmid pBR322 DNA. Pgap, glyceraldehyde-3-phosphate dehydrogenase promoter; Ac-A, acetylated N-terminal alanine of SOD and HB-SOD; Fr. A–D, synthetic DNA fragments.

3. RESULTS AND DISCUSSION

3.1. *Binding of HB-SOD to endothelial cell surface*

To test whether HB-SOD having a C-terminal basic domain might specifically bind to vascular endothelial cell surface, the radiolabeled enzyme samples were incubated with cultured endothelial cells. As shown in Fig. 2, HB-SOD bound to the cell surface of cultured endothelial cells. Since endothelial cells were enriched with acidic proteoglycans, such as heparan sulfate [20], the binding was also determined in the presence of heparin. As expected, binding of HB-SOD to endothelial cell surface was completely inhibited by the presence of heparin. Same concentration of chondroitin sulfate failed to inhibit the binding (data not shown). Under identical conditions, native SOD did not bind to vascular endothelial cells. Preliminary experiments revealed that intravenously injected HB-SOD predominantly localized on the lumenal surface of vascular endothelial cells [21]. Thus, superoxide radicals on the outer surface of vascular endothelial cells would efficiently be dismutated by membrane-bound HB-SOD. It has been well documented that subcutaneous injection of carrageenin, a potent inducer of local inflammation, increases the permeability of vascular endothelial cells and induces paw edema of the rat [22–25]. To test whether dismutation of superoxide radicals close to the outer surface of endothelial cells

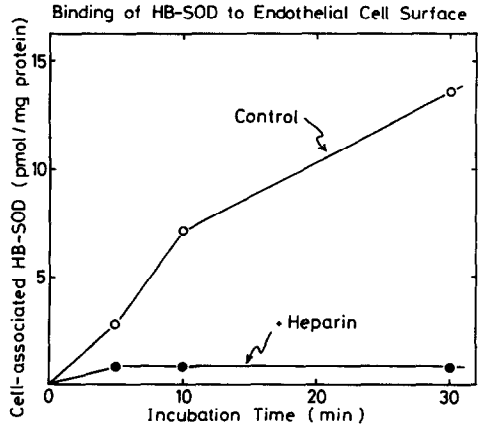

Fig. 2. Inhibition of HB-SOD binding to endothelial cell surface by heparin. Cultured endothelial cells from porcine aorta (2 days after confluence) were incubated in 2 ml Eagle's MEM [17] with 3 μg/ml of radioactive SOD or HB-SOD (1.2×10^4 and 1.4×10^4 cpm/μg protein, respectively) in the presence or absence of 560 μg/ml heparin at 0°C. At the indicated times after incubation, cells were washed with 3 ml of ice-cold MEM for 3 times and determined for cell-associated radioactivity. Open circles, HB-SOD; closed circles, HB-SOD + heparin; squares, native SOD.

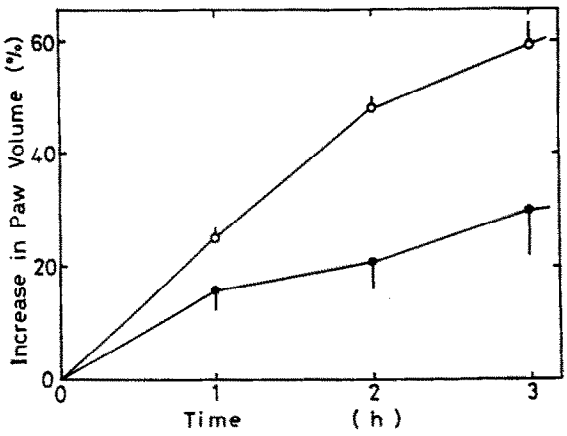

Fig. 3. Effect of HB-SOD on carrageenin-induced paw edema. Under light ether anesthesia, rats were intravenously administered 0.2 ml of saline (open circles) or 5 mg/kg of HB-SOD (closed circles). After 5 min, 2 mg of carrageenin in 0.1 ml saline was subcutaneously injected in the paw. At the inducated times after injection, the paw volume was measured by a plethysmometer (Unicom Model TK-101). Values show the increased paw volume (% of initial level) and are expressed as mean ± SD derived from 4–6 rats.

might protect tissues from being attacked by oxygen radicals, the effect of HB-SOD was tested on carrageenin-induced paw edema (Fig. 3). The volume of the paw increased with time after carrageenin injection. The paw edema was inhibited significantly by a single dose of HB-SOD. Thus, superoxide and/or its metabolite(s) might be responsible for the carrageenin-induced increase in vascular permeability.

To test whether reactive oxygens also play a critical role in vasogenic edema of other tissues, such as the brain, effect of HB-SOD was also observed on cold-induced brain edema. As shown in Fig. 4, HB-SOD markedly inhibited brain edema. Equimolar amounts of SOD, heat-denatured HB-SOD and the heparin-binding peptide failed to inhibit paw edema and brain edema at any conditions tested. These results suggested that superoxide radical and/or its metabolite(s) on the outer surface of vascular endothelial cells might play a critical role in the pathogenesis of vasogenic edema. Preliminary experiments revealed that HB-SOD also inhibited the lethal arrhythmias elicited by occluding the left anterior descending artery of the rat and decreased the mortality from 60% to 22%. Thus, superoxide radical and/its metabolite(s) might also be involved in various types of vasogenic diseases, such as ischemic myocardial injury.

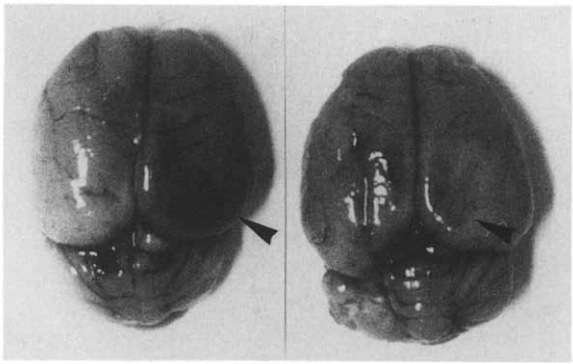

Fig. 4. Effect of HB-SOD on cold-induced brain edema. Under light ether anesthesia, animals were intravenously injected with 0.2 ml saline (left) or 5 mg/kg HB-SOD (right). After 5 min, cold-injury was induced at the right hemisphere of the brain (arrow) by applying a liquid-nitrogen-cold plasticine probe (20 g, 4 mm in diameter) to the right side of the bony skull for 20 s. Two hours after giving cold-injury, animals were intravenously injected with 0.2 ml of 1% Evan's blue. After 30 min, the animal was exsanguinated by bleeding and the brain was excised.

Since HB-SOD consisted of human type of Cu^{2+}/Zn^{2+}-SOD and the heparin-binding domain of EC-SOD and can easily be expressed in yeast with large amounts, HB-SOD may be useful for protecting vascular endothelial cells (and parenchymal cells) against oxygen toxicity without immunological complications. This methodology provides a new strategy for targeting enzymes and bioactive peptides to the subcellular site(s) where heparin-like proteoglycans are enriched and permits molecular studies on pathological events occurring at or near the vascular endothelial cell surface.

Acknowledgements: The authors express their gratitude to Ms Haruyo Hatanaka and Toshihiko Ashikari for their excellent technical assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Science, Literature and Culture of Japan.

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